Interaction of α_2-Macroglobulin with Trypsin, Chymotrypsin, Plasmin, and Papain[1]

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The interaction α_2-macroglobulin with four proteinases has been investigated by binding assays and by gel electrophoresis. At pH 7.65 the binding ratios of the proteinase–α_2-macroglobulin complexes were found to be 2:1 (trypsin and papain), 1.4:1 (chymotrypsin), and 1:1 (plasmin). The progressive decrease in the stoichiometry of the three seryl proteinase complexes was paralleled by a concomitant decrease in the proteinase-dependent specific cleavage of the α_2-macroglobulin peptide chains. Rate studies have shown that the relative rates of reaction of the proteinases with α_2-macroglobulin also varied greatly: papain > trypsin > chymotrypsin > plasmin. The data suggest that the ability of a proteinase to saturate the second proteinase binding site is a reflection of its ability to bind to α_2-macroglobulin and cleave the second pair of scissile α_2-macroglobulin peptide bonds before the α_2-macroglobulin has undergone the conformational change initiated by the formation of the 1:1 proteinase α_2-macroglobulin complex.

Human plasma α_2-macroglobulin is a plasma glycoprotein of approximately 720,000 daltons. The molecule is a tetramer composed of identical subunits linked in pairs by disulfide bonds. Two disulfide pairs associate noncovalently to form the native tetramer (1, 2).

Although α_2-macroglobulin is generally classified as a proteinase inhibitor, certain of its biochemical characteristics are strikingly different from those of other members of this group. Two such deviant properties are the ability of α_2-macroglobulin to bind an unusually wide variety of endopeptidases (seryl, cysteinyl, aspartyl, and metal proteinases) (3, 4) and the continued hydrolytic activity of α_2-macroglobulin-bound proteinases to small molecule, but not macromolecule, substrates (5). The binding of proteinase by α_2-macroglobulin is accompanied by a specific cleavage of the α_2-macroglobulin subunit chains (1, 2, 4) although in the absence of denaturants and small molecule thiols the α_2-macroglobulin–proteinase complex remains intact.

Literature values for proteinase:α_2-macroglobulin binding ratios fall between 1 and 2 mol proteinase bound per mole α_2-macroglobulin (3, 4, 6-9). The reported values show an unusual degree of variability. If we assume that α_2-macroglobulin intrinsically contains two binding sites for all proteinases (a fair assumption in view of

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the dimer characteristics of the molecule), the low binding ratios which have been reported for certain proteinases are presumably a reflection of ancillary events which restrict the ability of the α_2-macroglobulin to bind the second proteinase molecule.

The studies on the interaction of α_2-macroglobulin with trypsin, chymotrypsin, plasmin, and papain which are reported in this paper were designed to investigate the factors responsible for the variability in the binding ratios of these four α_2-macroglobulin–proteinase complexes.

MATERIALS AND METHODS

TPCK trypsin and 3× crystallized, salt-free α-chymotrypsin were obtained from Worthington Biochemical Corporation. Glu-plasminogen was prepared from fresh human plasma by affinity chromatography on a lysine-Sepharose 4B column (10) in the presence of aprotinin. Aprotinin-free plasminogen was converted to plasmin by incubation for 1 h at 25°C with urokinase-Sepharose 4B (11). The resultant plasmin preparation showed two bands on SDS[3]-polyacrylamide gel electrophoresis; a major plasmin band and a minor band which had the same mobility as Glu-plasminogen. Papain which had been purified by the method of Burke et al. (12) was a gift from Dr. Jules Shafer, Department of Biological Chemistry, University of Michigan Medical School.

Preparation of α_2-macroglobulin. Polyethylene glycol 6000 (J. T. Baker Chemical Company) was added to fresh plasma to give a concentration of 4.5%. The suspension was stirred for 30 min, centrifuged for 30 min at 5000g and the supernatant removed. Sufficient polyethylene glycol was then added to the supernatant to give a concentration of 10%. The resulting suspension was stirred for 30 min, and centrifuged as before. The precipitate was washed with 0.5 plasma volume of 10% polyethylene glycol in 0.15 M NaCl, recentrifuged, and the washed precipitate was dissolved in 30 ml of 0.15 M NaCl. These procedures were conducted in plastic containers (4°C).

The washed 4.5-10% precipitate was applied to a 5 × 85-cm gel filtration column containing LKB Ultrogel AcA 22. The column was eluted with 0.15 M NaCl, 0.015 M Tris buffer solution which had been adjusted to pH 7.0 with sodium carbonate. The α_2-macroglobulin eluate fraction (as determined by

trypsin-binding activity) was pooled, concentrated, and applied to a hemoglobin-Sepharose 4B affinity column which had been prepared by the general method of Klein and Mihaesco (13). The resultant haptoglobin-free α_2-macroglobulin fraction was pooled and concentrated, and contaminating immunoglobulins were removed from the α_2-macroglobulin fraction by preparative electrophoresis at pH 8.2 in a Brinkman Instrument electrophoretic separator, Brinkman Model FF. The α_2-macroglobulin fraction was pooled, concentrated, and reapplied to the Ultrogel AcA 22 column to obtain the purified α_2-macroglobulin preparation.

As determined by gel double diffusion against antisera to whole plasma, α_2-antitrypsin, antithrombin III, inter-α-trypsin inhibitor, immunoglobulin λ and κ chains, antichymotrypsin, haptoglobin, and albumin (Behring Diagnostics), the α_2-macroglobulin preparation was essentially free of other plasma proteins. SDS-gel electrophoresis of overloaded 4% polyacrylamide gels showed the presence of one major high-molecular-weight band with very faint traces of a lower-molecular-weight contaminant.

The molecular weight of α_2-macroglobulin was assumed to be 720,000 (14).

The α_2-macroglobulin proteinase interaction. The activities of the trypsin and plasmin preparations were determined by the active site titration method of Chase and Shaw (15, 16) using p-nitrophenyl-p'-guanidinobenzoate as substrate. The activity of the chymotrypsin preparations was determined by the active site titration method of Bender et al. (17) using p-nitrophenyl acetate as substrate. The activity of the papain preparation was determined in two assay systems; one procedure used N-α-benzoyl-L-arginine-p-nitroanilide (L-BAPNA) as substrate (18), the other 2,2'-dipyridyl disulfide (Aldrich Chemical Co.) as an active site titrant (19).

All proteinase concentrations given in this paper refer to "active" concentrations.

The stoichiometry of binding of trypsin to α_2-macroglobulin was determined by adding increasing amounts of α_2-macroglobulin to a constant amount of trypsin (α_2-macroglobulin:trypsin molar ratios of 0 to 1.07), adding soybean trypsin inhibitor to inactivate any unbound trypsin and then measuring the ability of the α_2-macroglobulin-bound trypsin to catalyze the hydrolysis of the low-molecular-weight substrate BAPNA (20).

The stoichiometry of the plasmin–α_2-macroglobulin complex was determined using an assay procedure identical to that employed in the assay of trypsin:α_2-macroglobulin reaction mixtures except that BAPNA was replaced by D-Val-Leu-Lys-NH-ϕ-NO$_2$, S-2251 (A. B. Kabi, Greenwich, Conn.). BAPNA is a relatively poor substrate for plasmin.

The stoichiometry of the chymotrypsin-α_2-macroglobulin complex was determined by a method sim-

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; L-BAPNA, N-α-benzoyl-L-arginine-p-nitroanilide; BTEE, N-benzoyl-L-tyrosine ethyl ester.

ilar to that employed in the trypsin-binding measurements. In this procedure, BAPNA was replaced by the chymotrypsin substrate N-benzoyl-L-tyrosine ethyl ester (BTEE) and soybean trypsin inhibitor was replaced by α_1-antitrypsin since chymotrypsin is not inactivated by soybean trypsin inhibitor but forms an inactive 1:1 complex with α_1-antitrypsin. The antitrypsin was prepared by the method of Bloom and Hunter (21).

The assay procedures used to measure the binding ratio of the α_2-macroglobulin–papain complex were somewhat different from those employed in the stoichiometry measurements on the other three proteinases since no macromolecular papain inhibitor was available. In one procedure, α_2-macroglobulin:papain reaction mixtures (papain:α_2-macroglobulin molar ratios of 0.8 to 15) were incubated with Azocoll (Calbiochem), an insoluble powdered cowhide to which a red dye had been covalently linked. Soluble Azocoll hydrolysis products were released in the presence of free but not α_2-macroglobulin-bound papain. The α_2-macroglobulin:papain stoichiometry was also determined by monitoring the ability of various α_2-macroglobulin:papain reaction mixtures to bind trypsin using BAPNA as substrate. This procedure depended on (i) the relatively low activity of papain toward BAPNA, the rate of the papain-catalyzed hydrolysis being less than one-fifth that of the trypsin-catalyzed hydrolysis, and (ii) the observation that saturating α_2-macroglobulin with papain resulted in a complex which was unable to bind trypsin.

SDS-gel electrophoresis under reducing and nonreducing conditions was performed by the method of Weber and Osborn (22).

RESULTS

The α_2-macroglobulin–trypsin complex. The binding of trypsin by α_2-macroglobulin was determined in the presence and absence of 7.5 mM EDTA or 3 mM EGTA at pH 5 (0.05 M acetate buffer), pH 7.0 (0.05 M phosphate buffer), pH 7.65 (0.05 M Tris), and pH 9.0 (0.05 M borate buffer) at 25°C. After a 60-min incubation period the pH of 3:1 trypsin:α_2-macroglobulin reaction mixtures was adjusted to 7.65 by the addition of 0.1 M Tris buffer. Control studies showed that α_2-macroglobulin was not inactivated by this long incubation period at pH 5–9. Excess soybean trypsin inhibitor was added to the reaction mixtures which were then assayed for α_2-macroglobulin-bound trypsin by the BAPNA assay procedure. In all cases the stoichiometry

of the complex was found to be 1.9–2.0:1 (Fig. 1).

The stoichiometry of binding was also investigated by SDS-polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol (Fig. 2). In the presence of SDS alone no proteolysis of α_2-macroglobulin to lower-molecular-weight species could be detected, even in reaction mixtures which contained excess trypsin. In the presence of SDS and mercaptoethanol, however, the ~180,000-dalton α_2-macroglobulin subunits were converted to ~90,000-dalton species and the amount of the ~90,000-dalton species increased with the trypsin concentration until the trypsin:α_2-macroglobulin ratio was approximately 2 at which point all the ~180,000-dalton subunits had been converted to the ~90,000-dalton species (Fig. 2). Complete hydrolysis of the four scissile α_2-macroglobulin peptide bonds thus accompanied binding of two molecules of trypsin by the α_2-macroglobulin.

The presence of Ca^{2+} (0–0.2 M $CaCl_2$) did not affect the stoichiometry of the α_2-macroglobulin–trypsin complex. It did however decrease the rate of hydrolysis of
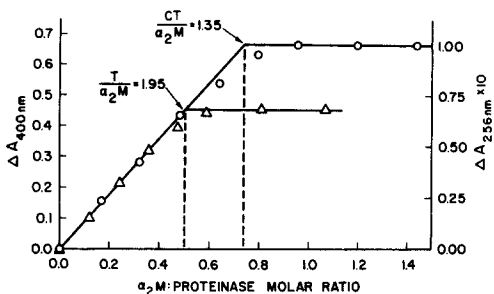

FIG. 1. Stoichiometry of α_2-macroglobulin–trypsin (α_2M-T) and α_2-macroglobulin-chymotrypsin (α_2M–CT) complexes. ($\triangle$) α_2M-Bound tryptic activity ($A_{400\ nm}$) of α_2M:T reaction mixtures in 0.025 M Tris, pH 7.65. Trypsin concentration = 0.5 μM; α_2M:T molar ratios = 0 to 1.07. Tryptic activity was determined by the BAPNA assay procedure in the presence of excess soybean trypsin inhibitor (STI). (O) α_2M-Bound chymotryptic activity ($A_{256\ nm}$) of α_2M:CT reaction mixtures in 0.025 M Tris, pH 7.65. Chymotrypsin concentration = 0.08 μM; α_2M:CT molar ratios = 0 to 1.45. Chymotryptic activity was determined by the BTEE assay procedure in the presence of excess α_1-antitrypsin.
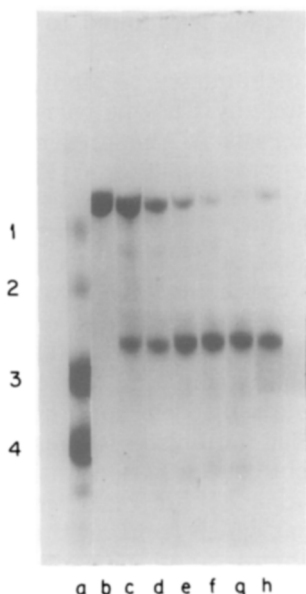
BAPNA as catalyzed by α_2-macroglobulin-bound trypsin, a situation in sharp contrast to the Ca^{2+}-activated hydrolysis of BAPNA by free trypsin.

Studies on the α_2-macroglobulin:trypsin interaction were extended to include the serial addition of nonsaturating amounts of trypsin to α_2-macroglobulin at pH 7.5; under such conditions the binding ratio did appear to decrease somewhat. For exam-

ple, when half-saturating amounts of trypsin were incubated with α_2-macroglobulin for from 1 min to 20 h at 25°C and excess trypsin then added, the average stoichiometry of the complex was 1.5:1 instead of the 2:1 obtained for the complex which had been prepared by one addition of excess trypsin. If one assumes that the rate of reaction of both trypsin molecules with native α_2-macroglobulin is the same (a reasonable assumption in view of the dimer characteristics of the molecule), the addition of an equivalent amount of trypsin to α_2-macroglobulin should result in a 25:50:25 mixture of 2:1, 1:1, and 0:1 trypsin–α_2-macroglobulin complexes. If excess trypsin is added later to this reaction mixture and if the 1:1 complex cannot bind trypsin, the resultant mixture should contain a 50:50 mixture of the 2:1 and 1:1 trypsin–α_2-macroglobulin complexes; that is, the overall binding ratio should be 1.5:1.

The α_2-macroglobulin–chymotrypsin complex. In our initial studies the binding ratio of the α_2-macroglobulin–chymotrypsin complex in 0.05 M Tris, pH 7.65, in the absence of Ca^{2+}, was found to be 1.4:1 (Fig. 1) and SDS-2-mercaptoethanol gel electrophoresis of such mixtures showed that only about two-thirds of the ~180,000-dalton subunits were cleaved to ~90,000-dalton subunits even in the presence of excess chymotrypsin (Fig. 3). To inhibit the generalized proteolysis of the α_2-macroglobulin which occurred during the 1-h, 25°C incubation of the chymotrypsin:α_2-macroglobulin reaction mixtures with SDS and mercaptoethanol in the absence of a small molecule inhibitor, toluene sulfonyl fluoride was added to the reaction mixtures prior to the addition of SDS and mercaptoethanol. Higher incubation temperatures (and hence shorter incubation times) could not be employed as they result in the cleavage of a specific, temperature-sensitive α_2-macroglobulin peptide bond (23). Furthermore when various nonsaturating amounts of chymotrypsin were incubated with α_2-macroglobulin at pH 7.65 for 10 min and excess chymotrypsin was then added, the stoichiometries of the complexes were significantly lower (minimum chymotrypsin:α_2-macroglobulin
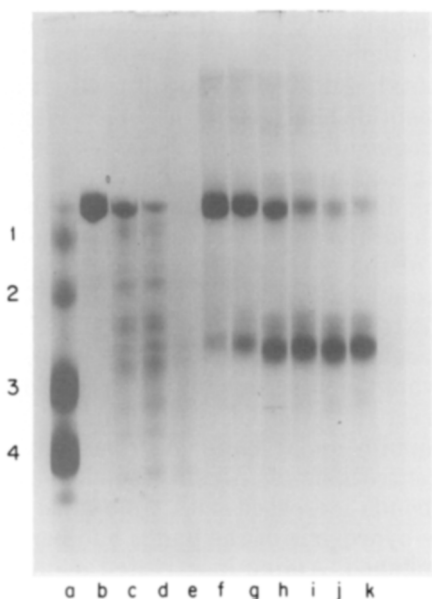
FIG. 3. Gel electrophoresis of α_2M:CT reaction mixtures in the presence of SDS and 2-mercaptoethanol. The samples were treated as described in Fig. 2. In gel b and gels f–k the α_2M:CT mixtures were incubated with 0.002 M toluene sulfonyl fluoride (TSF) for 30 min just prior to the addition of the SDS and 2-mercaptoethanol. (a) 1, Transferrin dimer; 2, BSA dimer, 3, transferrin monomer; 4, BSA monomer. (b) α_2M + TSF; (c–e) α_2M + 0.5, 1.0, and 1.5 eq of CT per mole α_2M; (f–j) α_2M + 0.35, 0.7, 1.05, and 1.4, and 4.2 eq of CT per mole α_2M + TSF; (k) as in gel i except that α_2M was incubated with CT for 24 h prior to the addition of the TSF. All other α_2M:CT incubations were for 10 min.

binding ratio = 1.2:1) than that of a comparable reaction mixture prepared by one addition of excess chymotrypsin (chymotrypsin:α_2-macroglobulin = 1.4:1) It was also observed that the saturated complex (binding ratio 1.4:1) did not bind trypsin even though the complex should not have been saturated with respect to trypsin binding sites.

The studies on the binding of chymotrypsin by α_2-macroglobulin were extended to include pH, EGTA (3 mM), EDTA (7.5 mM), and Ca^{2+} (20 mM), as variables. At pH 9.0, the stoichiometry of the complex was found to be 1.8:1 in the presence and absence of Ca^{2+}. In the presence of Ca^{2+} the stoichiometry of the complex at pH 7.65 was the same as that observed at

pH 9. In the absence of Ca^{2+} however, the stoichiometry decreased to 1.4:1. At lower pH values (pH 5) the stoichiometry was further depressed to a low of ~1:1. When an excess amount of an equimolar mixture of trypsin and chymotrypsin was quickly added to α_2-macroglobulin at pH 7.65 and the reaction mixture assayed for tryptic activity in the presence of excess soybean trypsin inhibitor, 80% of the bound proteinase was found to be trypsin (Fig. 4). It therefore appears that the lower stoichiometry of the chymotrypsin-α_2-macroglobulin complex is accompanied by a considerably slower rate of binding.

The α_2-macroglobulin-plasmin complex. As previously reported by Pochon (24) plasmin was found to form a 1:1 complex with α_2-macroglobulin (Fig. 5). When excess trypsin was added to a series of plasmin:α_2-macroglobulin reaction mixtures which varied in plasmin concentration and any excess free proteinase then inactivated by the addition of soybean trypsin inhibitor, it was observed that the trypsin-catalyzed hydrolysis of BAPNA decreased with increasing plasmin concentration and was essentially zero in reaction mixtures
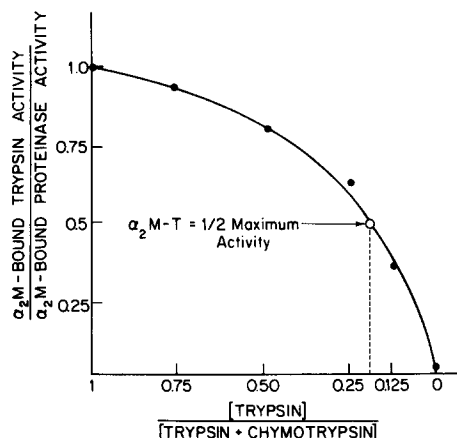
FIG. 4. The tryptic and total proteinase activities of α_2M:(T + CT) reaction mixtures. α_2M concentration = 0.25 μM; total proteinase:α_2M molar ratio = 4:1. The trypsin:total proteinase molar ratio varied from 0 to 1. α_2M-Bound tryptic activity was determined by the BAPNA assay procedure in the presence of excess STI, α_2M-bound chymotryptic activity by the BTEE assay procedure in the presence of excess α_1-antitrypsin. Buffer = 0.1 M Tris, pH 7.65.

FIG. 5. The interaction of plasmin with α_2M. (A) Various amounts of 5.6 μM plasmin were mixed with 1.0 ml of 0.05 M Tris, pH 7.6, and 0.1 ml of 1.1 μM α_2M. The volumes of the solutions were made up to 1.5 ml with 0.1 M NaCl. After 10 min 0.06 ml of 1 mg/ml STI was added to the reaction mixtures to inactiv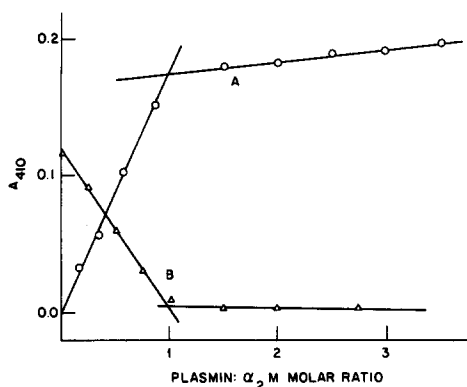ate unbound plasmin. 0.5 ml of S-2251 (substrate) was then added to the reaction mixtures. After 10 min the reaction was stopped by the addition of 1 ml of 33% acetic acid. (B) Reaction mixtures similar to those in A were prepared except that 0.04 ml of 1 mg/ml trypsin (excess) was added to the plasmin:α_2-macroglobulin reaction mixtures before the STI. The substrate in these experiments was DL-BAPNA and the reactions were stopped after 15 min by the addition of acetic acid. The data have been corrected for the slow hydrolysis of BAPNA by α_2M-bound plasmin.

that contained a plasmin:α_2-macroglobulin ratio of one or greater (Fig. 5). BAPNA is such a poor substrate for plasmin relative to trypsin that the α_2-macroglobulin-bound plasmin in the assay solution made a negligible contribution to the observed hydrolysis of the BAPNA.

Reducing SDS-gel electrophoresis showed that a maximum of about one-half of the α_2-macroglobulin chains could be cleaved by plasmin; all α_2-macroglobulin chains, however, were cleaved after the subsequent addition of excess trypsin to the saturated α_2-macroglobulin–plasmin complex.

This trypsin-catalyzed hydrolysis of the α_2-macroglobulin peptide chains of the saturated 1:1 plasmin–α_2-macroglobulin complex might appear to conflict with the binding data which showed that the saturated plasmin complex did not form a stable complex with trypsin.

Similar reducing SDS-gel electrophoresis studies, however, showed that trypsin catalyzed the cleavage of the four scissile peptide bonds of amine-inactivated α_2-macroglobulin, a species that does not form a stable complex with trypsin (25). The rate of this hydrolysis was slower than that of the hydrolysis that accompanied formation of the native α_2-macroglobulin–trypsin complex. Furthermore, we found that the extent of cleavage of the scissile α_2-macroglobulin peptide bonds of amine-inactivated α_2-macroglobulin was no longer a direct reflection of the trypsin: α_2-macroglobulin molar ratio. At relatively low concentrations of trypsin, the cleavage of the bonds increased with time until complete hydrolysis of the bonds had been accomplished. Amine-inactivated α_2-macroglobulin thus acted as a rather poor substrate for trypsin, rather than as an inhibitor.

Barrett (3, 26) has shown that the structural characteristics of amine-inactivated α_2-macroglobulin differ from those of the native molecule but appear similar to those of the α_2-macroglobulin–proteinase complexes. Like the amine-inactivated protein, the plasmin complex may therefore also act as a poor substrate and the trypsin-catalyzed hydrolysis of the labile α_2-macroglobulin peptide bonds in the plasmin complex may be completely unrelated to the formation of a stable complex between the 1:1 plasmin–α_2-macroglobulin complex and trypsin.

The rate of the α_2-macroglobulin:plasmin reaction was much slower than that of the α_2-macroglobulin:trypsin reaction at pH 7.65. When an equimolar mixture of plasmin and trypsin was quickly added to an α_2-macroglobulin solution (total proteinase:α_2-macroglobulin molar ratio = 4:1) and the reaction mixture assayed for trypsin and total proteinase activity after the addition of excess soybean trypsin inhibitor to inactive unbound proteinase, it was found that essentially all the α_2-macroglobulin-bound proteinase was trypsin. When the plasmin:trypsin molar ratio was increased to 5:1 (total proteinase:α_2-macroglobulin molar ratio = 12:1) about 5% of the α_2-macroglobulin-bound proteinase

was plasmin. Total α_2-macroglobulin-bound proteinase activity was determined using S-2251 as substrate since this is a good substrate for both proteinases.

No Ca^{2+} was present in any of the α_2-macroglobulin plasmin reaction mixtures.

The α_2-macroglobulin-papain complex. S-Methyl-papain solutions were activated in 100 mM 2-mercaptoethanol, 2.5 mM EDTA, pH 5.8, for 30 min (25°C) and passed down a Sephadex G-25 column to remove small molecule by-products. The column buffer was 0.15 M KCl, 2.5 mM EDTA, 50 μM 2-mercaptoethanol, pH 5.8.

Under our conditions of pH and buffer, the concentration of 2-mercaptoethanol must be 1 mM or greater to achieve full activation of *S*-methyl-papain. To retain the full activity of this activated papain at lower mercaptoethanol concentrations, the small molecule by-products of the activation reaction must be removed from the activated papain preparation. The final concentration of mercaptoethanol in papain:α_2-macroglobulin reaction mixtures must be 1 mM or lower to prevent loss of the proteinase-binding capacity of the α_2-macroglobulin.

When the Azocoll assay system was used to determine the free papain concentration in α_2-macroglobulin:papain reaction mixtures (pH 7.65) of varying papain:α_2-macroglobulin molar ratio, the stoichiometry of the saturated complex was found to be 2:1 (Fig. 6). It thus appeared that at pH 7.65 papain, like trypsin, can completely saturate both proteinase binding sites on α_2-macroglobulin. These results were supported and extended using a second method to determine the α_2-macroglobulin-papain stoichiometry. Papain and α_2-macroglobulin were mixed in varying proportions at pH 7.65 such that the papain:α_2-macroglobulin molar ratio varied from 0 to 5. After a 10-min incubation period excess trypsin was added to the reaction mixtures. Any unbound trypsin was then inactivated by the addition of soybean trypsin inhibitor. At low papain concentrations the binding of trypsin was evidenced by the ability of the complex to catalyze the hydrolysis of BAPNA. As the papain:α_2-macroglobulin molar ratio in-
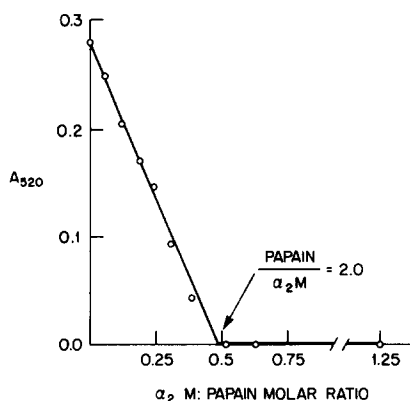

FIG. 6. The interaction of papain with α_2M. 5.8 $\times 10^{-10}$ mol of papain were mixed with zero to 7.4 $\times 10^{-10}$ mol of α_2-macroglobulin in 2.1 ml of a solution containing 50 mM Tris, 2.5 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 7.65. After 10 min 2 ml of this solution was added to 10 mg of powdered Azocoll. The mixture was incubated for 15 min after which time the reaction was stopped by the addition of 1.0 ml of 33% acetic acid. The 520-nm absorbance values shown in the figure were obtained by subtacting the absorbance of a control sample (a sample to which the acetic acid had been added before addition of the Azocoll) from that of the comparable reaction mixture.

creased, the hydrolysis of BAPNA decreased to a minimum at approximately a 2:1 molar ratio of papain to α_2-macroglobulin. At higher papain concentrations a slow increase in hydrolysis paralleled the increase in papain concentration. This slow increase resulted from the hydrolysis of BAPNA by papain since papain is not inactivated by soybean trypsin inhibitor. These data thus indicated that the saturated papain–α_2-macroglobulin complex was unable to bind trypsin.

The relative rates of formation of the α_2-macroglobulin–papain and α_2-macroglobulin–trypsin complexes were determined by quickly mixing equimolar amounts of papain and trypsin and adding excess proteinase to the α_2-macroglobulin solution at pH 7.65 in the presence of 1 mM 2-mercaptoethanol. These low mercaptoethanol concentrations did not measureably affect the interaction of trypsin with α_2-macroglobulin. Any unbound trypsin was then inhibited by the addition of excess soybean trypsin inhibitor. Bound and

unbound papain were inhibited by the addition of excess iodoacetate (10 mM). Iodoacetate did not depress the hydrolytic activity of trypsin nor did it diminish the proteinase-binding ability of α_2-macroglobulin. Any observed hydrolysis of BAPNA was thus due to α_2-macroglobulin-bound trypsin. In control experiments, the hydrolytic activity of trypsin-free, papain–α_2-macroglobulin complex in the presence of iodoacetate was found to be negligible. The rate of hydrolysis of BAPNA by the papain + trypsin:α_2-macroglobulin reaction mixtures was thus a measure of α_2-macroglobulin-bound trypsin. The data indicated that only about 10% of the α_2-macroglobulin-bound proteinase was trypsin. When the trypsin:papain molar ratio was increased to 10:1 approximately 50% of the bound proteinase was trypsin.

In parallel studies in which Azocoll was used to monitor the unbound papain concentration, the α_2-macroglobulin was also found to bind papain approximately 10 times faster than trypsin. In these studies any unbound trypsin was inactivated by the addition of soybean trypsin inhibitor; no iodoacetate was added to the reaction mixtures.

DISCUSSION

The recent studies of Barrett et al. (26, 27), of Sottrup-Jensen et al. (28–33), and of others have suggested that the interaction of α_2-macroglobulin with proteinases involves several events. The interaction is initiated by binding of the proteinase at its active site to the "bait" region of the α_2-macroglobulin peptide chain. This interaction results in the cleavage of a proteinase-labile peptide bond near the middle of the α_2-macroglobulin peptide chain (29, 32). Peptide bond cleavage is followed by a change in the conformation of the α_2-macroglobulin which becomes more compact. During or after this conformational change, the proteinase binds to a different site on the α_2-macroglobulin. This interaction does not involve the active site of the proteinase which remains catalytically active. This terminal interaction may involve the unique glutamyl–cysteinyl thioester linkage in the α_2-macroglobulin which apparently becomes much more reactive after either cleavage of the α_2-macroglobulin peptide chain or the conformational change has occurred.

This pathway has been based largely on data obtained on the interaction of trypsin with α_2-macroglobulin. Per se, it does not explain the differences which have been observed in the binding ratios of the various proteinase–α_2-macroglobulin complexes.

All available data show that at least one molecule of a reactive proteinase is bound in a stable complex by α_2-macroglobulin. The variability only arises in the extent to which a second proteinase molecule can be bound.

The binding and SDS–gel electrophoresis studies reported in this paper strongly suggest that there is a parallelism between the stoichiometry of the saturated complex and the extent to which the proteinase is able to cleave the scissile α_2-macroglobulin peptide bonds. Trypsin (stoichiometry = 2:1) cleaves all four of the scissile α_2-macroglobulin peptide bonds; chymotrypsin (pH 7.65, stoichiometry = 1.4:1) cleaves about two-thirds of the scissile bonds; plasmin (stoichiometry = 1:1) cleaves about half of the scissile bonds. In other words, if the second proteinase molecule cannot bind to and cleave the "bait" region of the two intact α_2-macroglobulin chains still present in the 1:1 proteinase–α_2-macroglobulin complex it is unable to bind to α_2-macroglobulin at the sites which characterize the stable, end-product complex.

The studies on the rate of reaction of the four proteinases with α_2-macroglobulin suggest that the stoichiometry of the saturated proteinase–α_2-macroglobulin complex is directly related to the rate of formation of the transient active site complex. Trypsin (stoichiometry = 2:1) is bound by α_2-macroglobulin 10 times slower than papain (stoichiometry = 2:1), 4 times faster than chymotrypsin (pH 7.65, stoichiometry = 1.4:1), and 100 times faster than plasmin (stoichiometry = 1:1).

If α_2-macroglobulin undergoes a major

conformational change upon interaction with a proteinase as proposed by Barrett (3), and if this "fast" form α_2-macroglobulin can no longer bind proteinase, as has been noted for trypsin by Van Leuven (34), it is reasonable to propose that the stoichiometry of binding for a particular proteinase will be related to its rate of binding to α_2-macroglobulin relative to the rate of the α_2-macroglobulin conformational change. Thus, if the rate of the initial proteinase:α_2-macroglobulin interaction is fast relative to that of the conformational change, the "native" conformation of α_2-macroglobulin will bind two molecules of proteinase. If the rate of the proteinase:α_2-macroglobulin interaction approaches that of the conformational change then only partial occupancy of the second binding site will occur since a proportion of the α_2-macroglobulin molecules will have undergone the conformational change before the second proteinase molecule can bind to the α_2-macroglobulin. If the rate of binding of proteinase is slow relative to that of the conformational change, only one proteinase molecule will be bound. For example, the rate of formation of the plasmin–α_2-macroglobulin complex is relatively slow and a stoichiometry of 1:1 is obtained for this complex.

This hypothesis can explain (i) the lower proteinase binding ratios that are associated with the slower chymotrypsin and plasmin reactions (relative to trypsin), (ii) the lower stoichiometry of partially saturated trypsin- and chymotrypsin-α_2-macroglobulin complexes to which excess trypsin or chymotrypsin had been added later, and (iii) the effect of pH on the stoichiometry of the chymotrypsin-α_2-macroglobulin complex but not of the trypsin complex. If the rate of the trypsin interaction is sufficiently faster than that of the conformational change, the relatively small changes in the rate of binding which are induced by varying the pH from 9 to 5 should not measurably affect the stoichiometry of the complex. If however the rate of binding of chymotrypsin to α_2-macroglobulin is sufficiently close to that of the conformational change, changes in pH could sufficiently alter the relative rates of

these two reactions such that the stoichiometry of the stable, end-product complex would be pH dependent.

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